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THE S. AUREUS FIBRINOGEN BINDING PROTEIN GENE

Field of the Invention

The invention relates to the isolation of the fibrinogen binding
20 protein gene from Staphylococcus aureus and to the use of the
fibrinogen binding protein and antibodies generated against it for
wound healing, blocking adherence to indwelling medical devices,
immunisation or diagnosis of infection.

25 ### Background of the Invention

In hospitalised patents Staphylococcus aureus is an important cause of
infections associated with indwelling medical devices such as catheters
and prostheses (Maki, 1982; Kristinsson, 1989) and non-device related
30 infections of surgical wounds. A recent significant increase in
isolates from European and US hospitals which are resistant to several
antibiotics and the potential threat of emergence of vancomycin
resistance in S. aureus has reinforced the importance of developing
alternative prophylactic or vaccine strategies to decrease the risk of
35 nosocomial infections due to S. aureus.

Initial localised infections can lead to more serious invasive
infections such as septicaemia and endocarditis. In infections

associated with medical devices, plastic and metal surfaces become
coated with host plasma and matrix proteins such as fibrinogen and
fibronectin shortly after implantation (Baier, 1977; Kochwa et al,
1977; Cottonaro et al, 1981). The ability of S. aureus to adhere to
5   these proteins is believed to be a crucial determinant for initiating
infection (Vaudaux et al, 1989, 1993). Vascular grafts, intravenous
catheters, artificial heart valves and cardiac assist devices are
thrombogenic and are prone to bacterial colonization. S. aureus is the
most damaging pathogen of such infections.

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Fibrin is the major component of blood clots and fibrinogen/fibrin is
one of the major plasma proteins deposited on implanted biomaterial.
There is considerable evidence that bacterial adherence to
fibrinogen/fibrin is of importance in initiation of device related
15   infection. (i) S. aureus adheres to plastic coverslips coated in vitro
with fibrinogen in a dose-dependent manner (Vaudaux et al, 1989) and to
catheters coated in vitro with fibrinogen (Cheung and Fischetti,
1990). (ii) The organism binds avidly via a fibrinogen bridge to
platelets adhering to surfaces in a model that mimics a blood clot or
20   damage to a heart valve (Herrmann et al., 1993). (iii) S. aureus can
adhere to cultured endothelial cells via fibrinogen deposited from
plasma acting as a bridge (Cheung et al., 1991). This suggests that
fibrinogen could have a direct role in promoting invasive
endocarditis. (iv) Mutants defective in a global regulatory gene sar
25   have reduced adherence to fibrinogen and have reduced infectivity in a
rat endocarditis infection model (Cheung et al., 1994). While this is
indicative of a role for adherence to fibrinogen in initiating
endocarditis it is by no means conclusive because sar mutants are
pleiotropic and could also lack other relevant factors.

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A receptor for fibrinogen often called the "clumping factor" is located
on the surface of S. aureus cells (Hawiger et al., 1978, 1982). The
interaction between bacteria and fibrinogen in solution results in
instantaneous clumping of bacterial cells. The binding site for
35   clumping factor of fibrinogen is located in the C-terminus of the gamma
chain of the dimeric glycoprotein. The affinity for the fibrinogen
receptor is very high (Kd 9.6 X 10^{-9} M) and clumping occurs in low
concentrations of fibrinogen. It is assumed that clumping factor also

promotes bacterial adhesion to solid-phase fibrinogen and to fibrin.

Clumping factor has eluded previous attempts at molecular characterisation. Reports of attempts to purify clumping factor
5   described molecules with molecular masses ranging from 14.3kDa to 420kDa (Duthie, 1954; Switalski, 1976; Davison and Sanford, 1982; Espersen et al., 1985; Usui, 1986; Chhatwal et al., 1987; Lantz et al., 1990) but none were followed up with more detailed analysis. Fibrinogen is often heavily contaminated with IgG and fibronectin and
10   unless specific steps were taken to eliminate them these studies must be suspect.

More recently it has been shown that S. aureus releases several proteins that can bind to fibrinogen (Boden and Flock, 1989, 1992,
15   1994; Homonylo McGavin et al., 1993). One of these is probably the same as the broad spectrum ligand binding protein identified by Homonylo McGavin et al., (1993). Another is coagulase (Boden and Flock, 1989), a predominately extracellular protein that activates the plasma clotting activity of prothrombin. Coagulase binds prothrombin
20   at its N-terminus and also interacts with fibrinogen at its C-terminus (McDevitt et al., 1992). However, a hypothesis that the cell-bound form of coagulase is the clumping factor was disproved when coagulase-defective mutants were shown to retain clumping factor activity (McDevitt et al., 1992). There is no evidence that the
25   fibrinogen binding region of any of these proteins is exposed on the bacterial cell surface and consequently there is no evidence that any is clumping factor.

Object of the Invention

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An object of the present invention is to obtain a minimal fibrinogen binding protein. A further objective is to obtain said protein by means of a genetic engineering technique by using e.g. a plasmid comprising a nucleotide sequence coding for said protein. A further
35   objective is to obtain said protein by chemical synthesis. An additional objective is to generate antisera against said protein.

Summary of the Invention

The present invention relates to an isolated fibrinogen binding protein gene from S. aureus, particularly the DNA molecule having the sequence
5   shown in Figure 2, or a substantially similar sequence also encoding S. aureus fibrinogen binding protein.

The invention also relates to hybrid DNA molecules, e.g. plasmids comprising a nucleotide sequence coding for said protein. Further the
10   invention relates to transformed host micro-organisms comprising said molecules and their use in producing said protein. The invention also provides antisera raised against the above fibrinogen binding protein and vaccines or other pharmaceutical compositions comprising the S. aureus fibrinogen binding protein. Furthermore the invention provides
15   diagnostic kits comprising a DNA molecule as defined above, the S. aureus fibrinogen binding protein and antisera raised against it.

By "substantially similar" is meant a DNA sequence which by virtue of the degeneracy of the genetic code is not identical with that shown in
20   Figure 2 but still encodes the same amino-acid sequence; or a DNA sequence which encodes a different amino-acid sequence which retains fibrinogen binding protein activity either because one amino-acid is replaced with another similar amino-acid or because the change (whether it be substitution, deletion or insertion) does not affect the active
25   site of the protein.

Drawings

The invention will be described further with reference to the drawings
30   in which there is shown:

Figure 1.   Adherence of S. aureus Newman strains to fibrinogen-coated PMMA coverslips.   The number of adherent bacteria is shown as a function of fibrinogen adsorbed on the coverslip.   The symbol for
35   Newman wild type is IIIIXIII.   Symbols for Newman mutant strains are as follows: mutant 1,-□-; mutant 2,-△-; mutant 3,-◇-; mutant 4,-▽-. Symbols for Newman mutants carrying pCF16 are as follows: mutant 1,-■-; mutant 2,-▲-; mutant 3,-◆-; mutant 4,-▼-.: The number of bacterial cells bound is shown as CFU (mean +/- range, n=2).   In points where

range bars are not visible, the bars are smaller than the symbols.

Figure 2. (A) Nucleotide and deduced amino acid sequence of the clfA
gene of Staphylococcus aureus strain Newman. The sequence has been
5   lodged in the EMBL Data Library under the accession number Z18852
SAUCF. Putative -35, -10, ribosome binding site (RBS) and
transcriptional stop regions are indicated on the nucleotide sequence.
For the ClfA protein, the start of the signal peptide (S), non repeat
region (A), repeat region (R), wall-spanning region (W) and membrane
10 spanning region (M) are indicated by horizontal arrows. The LPXTG
motif is underlined.
(B) Schematic diagram showing the domain organization of the ClfA
protein. S, signal peptide; A, non-repeat region; R, repeat region; W,
wall region;, M, membrane spanning region and +, positively charged
15 residues. The position of the LPXTG motif is indicated.

Figure 3. Proteins purified from E. coli TB1 expressing pCF17. A DNA
fragment corresponding to the N-terminal half of ClfA (residues 23-550;
Region A) was generated by PCR and cloned in-frame into the expression
20 vector pKK233-2 to generate pCF17. The N-terminal sequence was deduced
for the three fibrinogen binding proteins (105kDa, 55 kDa and 42kDa)
purified from an induced culture of E. coli carrying pCF17 (Table 1)
and the location of each with respect to the A domain and amino acids
represented are indicated. Recombinant proteins which possess
25 fibrinogen binding activity are denoted by ++.

Figure 4. Western affinity blotting analysis of fibrinogen binding
proteins from E. coli TB1 expressing pCF17. Samples were fractionated
by SDS-PAGE, transferred to nitrocellulose membranes and probed with
30 fibrinogen labelled with horseradish peroxidase (HRP). 100 micro g of
E. coli pCF17 induced lysate (Lane 1), 5 micro g of proteins purified,
from the fibrinogen-Sepharose column and 1 micro g of purified 42kDa
protein. Sizes are in kDa.

35 Figure 5. Inhibition of adherence of strain Newman Δ spa to
fibrinogen-coated PMMA coverslips by anti-ClfA sera and preimmune
sera. The symbol for anti Region A serum N2 is -■- and the symbol for
preimmune serum N2 is -□-. The symbol for anti Region RWM serum C2 is
-●-. The percentage inhibition is shown as mean +/- range, n=2. In

points where range bars are not visible, the bars are smaller than the symbols.

Figure 6. Localization of the fibrinogen binding domain of ClfA. DNA
5 fragments corresponding to the different segments of clfA were
generated by PCR and cloned in-frame into the fusion protein expression
vector pGEX-KG. ClfA truncates were expressed as fusion proteins with
glutathione S-transferase. The location of the clfA gene fragments,
the amino acids represented and the length of the protein amplified are
10 also indicated. The properties of the recombinant proteins are
indicated. Proteins were assessed for (a) ability to bind to
fibrinogen in the affinity blotting assay (binds fg), (b) the ability
of lysates to inhibit the clumping of bacteria in soluble fibrinogen
(inhibits clumping), (c) the ability of lysates to inhibit the
15 adherence of bacteria to solid-phase fibrinogen (inhibit adherence),
and (d) the ability of lysates to block neutralising antibodies (blocks
Abs). ++, positive reaction; -, negative; ND, not done.

Figure 7. (A) Inhibition of adherence of S. aureus Newman to
20 fibrinogen-coated coverslips by lysates containing ClfA truncates.
Symbols are E. coli pCF24 uninduced lysate -△-, E. coli pCF24 induced
lysate -▲- E. coli pCF25 uninduced lysate -□-, E. coli pCF25 induced
lysate -■-. The percentage inhibition is shown as mean +/- range,
n=2. In points where range bars are not visible, the bars are smaller
25 than the symbols.
(B) Inhibition of adherence of S. aureus Newman to fibrinogen-coated
coverslips by lysates containing ClfA truncates. Symbols are E. coli
pCF27 lysate -■-, E. coli pCF28 lysate-●-, E. coli pCF29 lysate -▲-,
E. coli pCF30 lysate -▼- , E. coli pCF31 lysate -◆-. The percentage
30 inhibition is shown as mean +/- range, n=2. In points where range bars
are not visible, the bars are smaller than the symbols.

Figure 8. Western immunoblotting of ClfA proteins.
Proteins released from the cell wall of S. aureus strains Newman (lane
35 3) and Newman clfA (lane 4), and proteins expressed by E. coli TB1 pCF3
(carrying the cloned clfA gene, lane 2) and by E. coli TB1 without the
plasmid (lane 1), were studied by Western immunoblotting with anti-ClfA
antibodies. Approximately 100 micro g of protein was loaded for each
sample. Sizes are in kDa.

Figure 9. $\underline{S}$. $\underline{aureus}$ strains were studied by immunofluorescence with anti-ClfA N2 serum. Newman Δ $\underline{spa}$::Tc^r cells (+) and Newman Δ $\underline{spa}$::Tc^r $\underline{clfA}$::Tn$\underline{917}$ cells (-).

5 Figure 10. Adherence of $\underline{S}$. $\underline{aureus}$ Newman strains to PMMA coverslips coated $\underline{in}$ $\underline{vitro}$ with fibrinogen. The number of adherent bacteria is shown as a function of fibrinogen adsorbed on the coverslip. The symbols are, Newman wild type, -O- ; Newman $\underline{clfA}$::Tn$\underline{917}$, -●-. The number of bacterial cells bound is shown as c.f.u. (mean +/- range,

10 n=2). In points where range bars are not visible, the bars are smaller than the symbols.

Figure 11. Adherence of $\underline{S}$. $\underline{aureus}$ Newman strains onto segments of $\underline{ex}$ $\underline{vivo}$ polymer tubing exposed to canine blood. Adherence was tested to

15 both $\underline{ex}$ $\underline{vivo}$ polyvinylchloride (PVC) and to $\underline{ex}$ $\underline{vivo}$ polyethylene (PE). The symbols are, Newman wild type, -O-; Newman $\underline{clfA}$::Tn$\underline{917}$, -●-. The number of bacterial cells bound is shown as c.f.u. (mean +/- range, n=2). In points where range bars are not visible, the bars are smaller than the symbols.

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Figure 12. Adherence of $\underline{S}$. $\underline{aureus}$ 8325-4 strains onto segments of $\underline{ex}$ $\underline{vivo}$ polymer tubing exposed to canine blood. Adherence was tested to both $\underline{ex}$ $\underline{vivo}$ polyvinylchloride (PVC) and to $\underline{ex}$ $\underline{vivo}$ polyethylene (PE). The symbols are: 8325-4 wild type, -□-; 83254 $\underline{clfA}$::Tn$\underline{917}$, -■-; 8325-4

25 $\underline{clfA}$::Tn$\underline{917}$ (pCF4), -◪-. The number of bacterial cells bound is shown as c.f.u. (mean =/- range, n=2). In points where range bars are not visible, the bars are smaller than the symbols.

Cloning and sequencing the clumping factor gene

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In view of the difficulties mentioned above with (i) obtaining pure fibrinogen, (ii) the discrepancies in reported molecular weight of "clumping factor" and (iii) the diversity of different fibrinogen binding proteins, a different approach was taken to identify the

35 clumping factor gene involving isolating insertion mutants that inactivated the clumping phenotype. This has been described in detail by McDevitt $\underline{et}$ $\underline{al}$., (1994).

Transposon Tn917 (Tomich et al., 1980) was used to generate insertion
mutants that eliminated the fibrinogen clumping phenotype of S. aureus
strain Newman. The temperature sensitive plasmid pTV1ts which carries
Tn917 (Youngman, 1985) was transferred into strain Newman and several

5   transposon insertion banks were isolated by growing cultures at 43⁰
in broth containing erythromycin (to select for Tn917 after plasmid
elimination). Cultures of the banks were mixed with fibrinogen, the
agglutinated cells were removed and the surviving cells in the
supernatants were screened for clumping factor-deficient mutants. Four

10   mutants were isolated from separate banks. The Tn917 elements were
transduced into a wild-type Newman host with phage 85. In each case
all the transductants screened had inherited the clumping factor
deficiency proving that the Tn917 insertions caused the mutant
phenotypes. The clumping factor mutants expressed the same level of

15   coagulase as the wild-type strain, further supporting the conclusion
that clumping factor and coagulase are distinct entities.

The mutants were analyzed by Southern hybridization using an internal
fragment of Tn917 as a probe in order to identify HindIII junction

20   fragments comprising transposon and flanking chromosomal sequences. A
junction fragment from one mutant was cloned using standard techniques
in plasmid vector pUC18 (Yanisch Perron et al., 1985). A fragment
comprising only chromosomal DNA flanking the transposon was isolated
from this plasmid and used in turn as a probe in a Southern blot of

25   genomic DNA of Newman wild-type and each of the mutants. A HindIII
fragment of 7kb that hybridized in Newman wild-type was missing in each
of the mutants. Genomic DNA of Newman wild-type was cleaved with
HindIII and ligated with plasmid pUC18 cut with the same enzyme and
transformed into E. coli TBI (Yanisch-Perron et al., 1985).

30   Transformants were screened by colony hybridisation using the junction
fragment probe. Plasmid pCF3 (pUC18 carrying the 7kb HindIII fragment)
was isolated. Plasmid pCF3 was deposited at the
on   under the Accession No.   , such
deposit complying with the terms of the Budapest Treaty.

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The 7kb HindIII fragment was subcloned into pCL84, a single copy
non-replicating vector which integrates into the chromosome of S.
aureus (Lee et al., 1991), forming pCF16. pCF16 was transformed into S.
aureus strain CYL316 (Lee et al., 1991) selecting for tetracycline

resistance. The integrated plasmid was then transduced with phage 85 into each of the Newman clf mutants. In a microtitre clumping assay the Newman mutants were completely devoid of activity even at the highest concentrations of fibrinogen, whereas the wild-type had a titre

5 of 2048 and could interact productively with very low concentrations of fibrinogen. The integrated single copy plasmid pCF16 restored the clumping activity of each of the mutants to the same level as that of the parental strain. Thus the HindIII fragment must express a functional protein which complements the clumping deficiency of the

10 mutants.

S. aureus Newman adhered to solid-phase fibrinogen coated onto polymethylmethacrylate (PMMA) coverslips in a concentration dependent manner (Figure 1). Each clf mutant showed drastic reduction in

15 adherence. This was restored to the level of the parental strain by pCF16. This data shows that the ability of Newman to form clumps in soluble fibrinogen correlates with bacterial adherence to solid-phase fibrinogen.

20 Fragments from the 7kb HindIII fragment in pCF3 were subcloned into pGEM7 Zf(+) (Promega). The smallest fragment which still expressed the fibrinogen binding activity was a 3.5kb HindIII-KpnI fragment. The DNA sequence of this fragment was obtained using standard techniques and has been lodged in the EMBL Data Library under the accession number

25 Z18852 SAUCF. A single open reading frame of 2799 bp was identified (Figure 2A). The orf is called clfA and the gene product the ClfA protein. The predicted protein is composed of 933 amino acids (molecular weight 97,058 Da). A putative signal sequence of 39 residues was predicted. The predicted molecular weight of the mature

30 protein is 92kDa. Following the signal sequence is a region of 520 residues (Region A) which precedes a 308 residue region (region R) comprising 154 repeats of the dipeptide serine-aspartate (Figure 2A and 2B). The C terminus of ClfA has features present in surface proteins of other Gram positive bacteria (Schneewind et al., 1993) that are

35 responsible for anchoring the protein to the cell wall and membrane: (i) residues at the extreme C-terminus that are predominantly positively charged, (ii) a hydrophobic region which probably spans the cytoplasmic membrane and (iii) the sequence LPDTG which is homologous to the consensus sequence LPXTG that occurs in all wall-associated

proteins of Gram positive bacteria. This strongly suggests that ClfA
is a wall-associated protein and that the N terminal part is exposed on
the cell surface.

5   It is not obvious from the primary structure of ClfA or by comparison
of ClfA with other ligand binding proteins of S. aureus (fibronectin
binding protein, Signas et al., 1989; collagen binding protein, Patti
et al., 1992) which part of ClfA interacts with fibrinogen.

10 **Results**

(1) Purifying the N-terminal half of the fibrinogen receptor (ClfA)

A DNA fragment corresponding to the N-terminal half of ClfA (residues
15   23-550; Region A) was generated by polymerase chain reaction (PCR) and
cloned in-frame into the expression vector pKK233-2 (Amann and Brosius,
1985) to generate pCF17 (Figure 3). Expression of recombinant Region A
was induced by adding isopropyl Beta-D-thiogalactoside (IPTG) to
exponential cultures. Induced cultures contained two proteins of
20   105kDa and 55kDa which reacted with fibrinogen in a Western ligand
blotting assay (Figure 4, lane 1). A fibrinogen-Sepharose 4B column
was made by the method recommended by the manufacturer (Pharmacia). A
sample of an induced culture containing these fibrinogen binding
proteins was passed into the fibrinogen Sepharose 4B column. Four
25   proteins were eluted: -105kDa, 55kDa, 42kDa and 75kDa (trace amounts).
In a separate purification experiment, the 42kDa protein was purified
to homogeneity. Only the 105kDa, 55kDa and 42kDa proteins bound to
fibrinogen in the Western ligand blotting assay (Figure 4, lanes 2
and 3). The N-terminal sequence of these proteins was determined
30   (Table 1). The 75kDa protein was present in trace amounts (1-2pmoles)
and is not related to ClfA. The three predominant proteins bound to
fibrinogen in the Western blotting assay and are related to the region
A (see Figure 3). The 105kDa protein represents the intact Region A
while the 55kDa and 42kDa proteins are breakdown products. The
35   apparent molecular weights of the native region A and breakdown
products of region A are much higher than that predicted from the DNA
sequence (Table 1).

(2) Antibodies to the Region A of the ClfA protein (residues 23-550)

A rabbit was immunised with 30 micro g of a mixture of the 105kDa,
75kDa, 55kDa and 42kDa proteins along with Freund's complete adjuvant.
5   The immune sera was called N2. One rabbit was also immunised with 18
micro g of the purified 42kDa ClfA truncate and the immune serum for
this was called N3. Bacterial interaction with fibrinogen can be
measured by a quantitative clumping titration assay (Switalski, 1976).
In this assay, doubling dilutions of a fibrinogen solution (1mg/ml) are
10 mixed in a microtitre dish with a suspension of 2 X 10^7 cells for 5
min with gentle shaking. A standard clumping concentration of
fibrinogen was defined as 2X the titre. To this was added varying
amounts of the anti-ClfA serum to measure the minimum inhibitory
concentration that stops the clumping reaction (Table 2). Both N2 and
15 N3 sera were potent inhibitors of the clumping of bacteria. Preimmune
sera did not inhibit the clumping of bacteria. N2 sera also had a
potent inhibitory activity on bacterial adhesion to surface-bound
fibrinogen in the coverslip assay (McDevitt et al., 1992, 1994),
expressing 95% inhibition at 1 micro g protein/ml (Figure 5).
20 Preimmune sera did not have any inhibitory activity even at a protein
concentration of 100 micro g/ml (Figure 5). In addition, antisera
raised against regions R, W and M (C2) (see section 4 below) failed to
inhibit adherence even at 100 micro g/ml (Figure 5).

25 (3) Localisation of the fibrinogen binding domain of the ClfA protein

DNA fragments corresponding to the Region A of ClfA (residues 23-550)
and C terminal regions R,W and M (residues 546-933) were generated by
PCR (standard conditions,) and cloned in-frame into the fusion protein
30 expression vector pGEX-KG (Guan and Dixon, 1991) to generate pCF24 and
pCF25 respectively (see Figure 6). These ClfA truncates were expressed
as fusion proteins with glutathione S-transferase. An induced lysate
of E. coli pCF24 (residues 23-550) expressed a fusion protein that
bound to fibrinogen in a Western affinity blotting assay with
35 peroxidase labelled fibrinogen (Figure 6). In addition, this lysate
inhibited the clumping of bacteria with soluble fibrinogen in the
clumping assay (Table 3 and Figure 6) and also inhibited the adherence
of bacteria to immobilised fibrinogen in the coverslip assay in a dose
dependent fashion (Figure 7A). A lysate of E. coli pCF25 (residues

546-933) induced with IPTG expressed a fusion protein that failed to
bind to fibrinogen in the Western blotting assay (Figure 6). In
addition, this lysate did not inhibit the clumping of bacteria in the
clumping assay (Table 3) and did not inhibit adherence to immobilized

5 fibrinogen in the adherence assay (Figure 7A). Uninduced lysates from
both pCF24 and pCF25 failed to inhibit both clumping and adherence
(Table 3 and Figure 7A).

A synthetic peptide (SDSDSDSDSDSDGGGC) designed to mimic the C-terminal

10 repeat region of ClfA failed to inhibit the clumping of bacteria in the
clumping assay when up to 107 micro g/ml was tested. In addition, the
synthetic peptide failed to inhibit the adherence of bacteria in the
adherence assay even at a concentration of 100 micro g/ml. Taken
together, this data suggests that the fibrinogen binding domain of ClfA

15 is located in the A domain rather than in the regions R, M, and W. It
confirms the data in Table 1 which dealt with purifying fibrinogen
binding proteins expressed from pCF17 and also suggests that an
N-terminal ClfA protein can act both as a potent inhibitor of cell
clumping in fibrinogen and also as a potent inhibitor of the adherence

20 of bacteria to fibrinogen coated surfaces.

The fibrinogen binding domain was further localised within region A.
Segments of region A were amplified by PCR and cloned into the pGEX-KG
vector. Lysates from IPTG-induced cultures were examined for the

25 presence of fibrinogen binding fusion proteins, for the ability to
inhibit the clumping of bacteria in the fibrinogen clumping assay and
for the ability to inhibit adherence to immobilised fibrinogen in the
adherence assay. The fusion protein of pCF31 (residues 221-550) was
the smallest truncate that still expressed a fibrinogen binding

30 activity (Figure 6). It is almost identical in composition to the
purified 42kDa protein (residues 219-550) described above. The fusion
proteins from pCF27, pCF28, pCF29 and pCF30 all failed to bind to
fibrinogen in the Western affinity blotting assay, despite reacting
with antibodies generated against the A domain of ClfA (Figure 6). In

35 addition, a lysate containing the fusion protein expressed by pCF31 was
the only one to inhibit the fibrinogen clumping reaction (Table 3) and
to inhibit the adherence of bacteria to immobilised fibrinogen in the
adherence assay (Figure 7B). These results suggest that the fibrinogen
binding site is quite extensive or that its correct conformation is

determined by flanking sequences.

An antibody neutralisation assay was adopted to help localise further
the active site within residues 221-550. This assay was conducted with
5   a protein A negative deletion mutant of S. aureus strain Newman (Patel
et al., 1987) to prevent non specific reaction with IgG. Polyclonal
antibodies raised against the A region of ClfA (N2) inhibited the
clumping of bacteria in soluble fibrinogen (see section 2 above). In
the standard clumping assay with the clumping concentration at 2X the
10 titre, the concentration of lysates that blocked the inhibitory
activity of 4.68 micro g of serum (2X the inhibitory concentration,
Table 2) was determined. The lysates containing ClfA fusion proteins
were assayed for their ability to neutralise the inhibiting activity of
the antibodies. Truncates containing the active site might be able to
15 adsorb out antibodies generated against the active site and thus
neutralise the blocking effect on cell clumping. The lysates
containing proteins expressed by pCF24 and pCF31 neutralised the
inhibiting activity of the antibodies while a lysate containing the
fusion protein expressed by pCF25 (Region R,W and M) did not inhibit
20 (Table 4). Lysates containing small fusion proteins expressed by pCF30
were able to neutralise the inhibiting activity of antibodies while
lysates containing fusion proteins expressed by pCF27 and pCF29 had no
such activity (Table 4). Taken together this suggested that the active
site is located in a 218 residue region between residues 332 and 550.

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(4) Antibodies to the C-terminal half of the ClfA protein (residues
546-933)

The fusion protein present in a lysate of E. coli pCF25 (residues
30 546-933) induced with IPTG was purified to homogeneity by using
glutathione sepharose-affinity chromatography as described by Guan and
Dixon, (1991). A rabbit was immunised with 20 micro g of the fusion
protein along with Freund's complete adjuvant. The immune sera was
called C2. This serum failed to inhibit the clumping of bacteria in
35 the clumping assay (Table 2) and also failed to inhibit bacterial
adhesion to surface bound fibrinogen in the coverslip assay even at 100
micro g/ml (Figure 5).

(5) Identification of the native fibrinogen receptor

Proteins released from the cell wall of S. aureus strains and a lysate
of E. coli expressing the cloned clfA gene were studied by Western
5   immunoblotting with anti ClfA antibodies in order to identify ClfA
protein(s). A lysate of E. coli TB1 (pCF3) (carrying the cloned clfA
gene) contained several immunoreactive proteins (Figure 8, lane 2).
The largest of these was ca. 190kDa. The smaller proteins are probably
derivatives caused by proteolysis. S. aureus strain Newman also
10   expresses a ca. 190kDa immunoreactive protein (Figure 8, lane 3). A
smaller immunoreactive protein of ca. 130kDa was also detected and is
probably also caused by proteolysis. Despite the presence of protease
inhibitors and studying proteins released from cells harvested at
different stages in the growth cycle (from mid-exponential to late
15   stationary), two proteins of these sizes were always present (data not
shown). Both proteins were absent in extracts of the clumping factor
negative transposon insertion mutant of Newman (Figure 8, lane 4)
indicating that they are products of the clfA gene.

20   Previously we reported the size of the ClfA protein to be ca. 130kDa
(McDevitt et al., 1994) in an affinity blotting assay with fibrinogen
and peroxidase labelled anti-fibrinogen antibodies. Our current
immunoblotting assay is much more sensitive than the affinity blotting
assay. In addition, we now know that the ClfA protein is very
25   sensitive to degradation. Indeed the predominant immunoreactive
protein detected in samples from both E. coli TB1 (pCF3) and S. aureus
strain Newman which have been frozen and thawed more than twice is
130kDa indicating that the ca. 190kDa protein is labile (data not
shown). Thus, the ca. 130kDa protein detected in the affinity blotting
30   assay is most probably a smaller derivative of ClfA. The apparent size
of the native ClfA protein of strain Newman appears to be ca. 190kDa.
This is double that predicted from the DNA sequence, but this might be
due to the unusual structure and is consistent with the aberrantly high
apparent molecular weight of recombinant proteins (Table 1). The
35   recombinant N-terminal Region A protein expressed by E. coli pCF17 also
had an unexpectedly high apparent molecular weight.

(6) <u>Surface localization of the ClfA protein by immunofluorescent</u>
<u>microscopy</u>

Anti-ClfA region. A sera (N2) was used to confirm that Region A of ClfA
5 is exposed on the bacterial cell surface. Protein A-deficient mutants
of Newman and Newman <u>clf</u>A::Tn<u>917</u> (clumping factor transposon insertion
mutant) were isolated by transducing the Δ<u>spa</u>::Tc^r mutation from
8325-4 Δ <u>spa</u>::Tc^r to strains Newman and Newman <u>clf</u>A::Tn<u>917</u> using
phage 85. Protein A-deficient mutants were used to prevent
10 non-specific interaction with rabbit IgG. Cells from overnight
cultures of strains Newman Δ <u>spa</u>::Tc^r and Newman Δ<u>spa</u>::Tc^r
<u>clf</u>A::Tn<u>917</u> were diluted to As60 = 0.6-1.0 and fixed to glass slides
using gluteraldehyde. The slides were then incubated in anti-ClfA
region A serum (N2, 1 in 200) followed by fluorescein conjugated swine
15 anti-rabbit serum (Dakopatts, 1 in 40). The cells were studied for
fluorescence by microscopy (Nowicki <u>et al</u>., 1984). Newman Δ
<u>spa</u>::Tc^r cells fluoresced while Newman Δ <u>spa</u>::Tc^r <u>clf</u>A::Tn<u>917</u>
cells did not (Figure 9). This confirmed that region A of ClfA is
exposed on the cell surface of wild-type Newman and that this ClfA
20 protein is absent in the clumping factor deficient mutant.

(7) <u>Role of the fibrinogen receptor in adherence to in vitro- and ex</u>
<u>vivo-coated polymeric biomaterials</u>

25 A mutant of strain Newman defective in the clumping factor
(<u>clf</u>A1::Tn<u>917</u>) and a complemented mutant bearing pCF16 were studied for
adherence properties to biomaterials coated <u>in vitro</u> with fibrinogen
and to <u>ex vivo</u> biomaterial. A canine arteriovenous shunt has been
developed as a model to study plasma protein adsorption onto
30 intravenous catheters from short-term blood-biomaterial exposures and
to identify host proteins promoting adhesion of <u>Staphylococcus aureus</u>
(Vaudaux <u>et al</u>., 1991).

<u>S</u>. <u>aureus</u> strain Newman adheres strongly (in a concentration dependent
35 fashion) to polymethylmethacrylate (PMMA) coverslips coated <u>in vitro</u>
with canine fibrinogen (Figure 10). In contrast, the fibrinogen
receptor mutant was significantly defective (>95%) in its ability to
adhere to the canine fibrinogen coated coverslips (Figure 10). In the

ex vivo model, either polyethylene or polyvinyl chloride tubing was
exposed to canine blood for 5, 15 or 60 min at a flow rate of 300
ml/min, then flushed in phosphate buffered saline (PBS), cut into 1.5
cm segments and preincubated in 0.5% albumin in PBS to prevent

5 non-specific staphylococcal attachment. Then, each segment was
incubated with 4 x 10^6 CFU/ml of [3H]thymidine-labelled S. aureus for
60 min at 37°C in an in vitro adherence assay. When compared with the
wild-type strain Newman, the fibrinogen receptor mutant strain showed a
strong decrease (>80%) in attachment to ex vivo polyvinyl chloride and

10 polyethylene tubings (Figure 11). In addition, strain 8325-4 (which
binds poorly to fibrinogen-coated coverslips in vitro and to the ex
vivo polymer tubings) showed a significant increase in its ability to
adhere to the two different ex vivo polymer tubings when complemented
with a plasmid (pCF4) expressing the fibrinogen receptor gene (Figure

15 12).

The data shows that fibrinogen is the major plasma protein in a
short-term blood material interaction to promote staphylococcal
adherence and the possession of the fibrinogen receptor is a major

20 determinant in the ability of S. aureus to adhere to ex vivo
biomaterials.

(8) Role of the fibrinogen receptor in the pathogenesis of experimental
endocarditis

25

S. aureus strain Newman, the fibrinogen receptor mutant strain of
Newman (clfA::Tn917) and a fibrinogen receptor mutant complemented with
the clfA+ integrating plasmid pCF16 were compared in a previously
described model of experimental endocarditis (Garrison and Freedman,

30 1970). This rat model investigates the early events in experimental
endocarditis with catheter-induced aortic vegetations (Veg). Groups of
>/-8 rats were challenged with an inoculum that resulted in 90% of
vegetations being colonised by the wild-type organism (ID90). Animals
were injected intravenously with the same inocula of Newman clfA and

35 Newman clfA (pCF16). Animals were killed 12 hours after inoculation
and quantitative cultures of the blood, spleen and Veg were performed.
Table 5 shows the percentage of rats infected.

The data show that a mutant lacking the fibrinogen receptor was significantly less able to infect the catheter-induced aortic vegetations (decrease of 49%) when compared with the wild type strain Newman. In addition, the complemented strain had restored

5   infectivity. The fact that all three strains infected the spleens with similar numbers suggests that the presence or absence of the fibrinogen receptor interfered specifically with bacterial colonisation of the catheter-induced aortic vegetation.

10   This model strongly implicates the fibrinogen receptor as an important adhesin in the pathogenesis of S. aureus endocarditis and other cardiovascular infections associated with intravenous catheters, artificial heart valves and intravenous shunts.

15   **Uses of the invention**

1.   The fibrinogen binding protein or fragment containing the fibrinogen binding region can be used as a vaccine to protect against human and animal infections caused by S. aureus. For example, the

20   fibrinogen binding protein or fragment containing the fibrinogen binding region can be used as a vaccine to protect ruminants against mastitis caused by S. aureus infections.

2.   Polyclonal and monoclonal antibodies raised against the fibrinogen

25   binding protein or a fragment containing the fibrinogen binding domain can be used to immunise passively by intravenous injection against infections caused by S. aureus.

3.   The fibrinogen binding protein or an active fragment can be

30   administered locally to block S. aureus from colonising and infecting a wound.

4.   The antibody against the fibrinogen binding protein can be administered locally to prevent infection of a wound.

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5.   The fibrinogen binding protein or an active fragment or antibodies against the fibrinogen binding protein can be used to block adherence of S. aureus to indwelling medical devices such as catheters,

replacement heart valves and cardiac assist devices.

6.   The fibrinogen binding protein or an active fragment or antibodies against the fibrinogen binding protein can be used in combination with other blocking agents to protect against human and animal infections caused by S. aureus.

7.   The fibrinogen binding protein can be used to diagnose bacterial infections caused by S. aureus strains. The fibrinogen binding protein can be immobilised to latex or Sepharose (Trade Mark), and sera containing antibodies are allowed to react; agglutination is then measured.

8.   The fibrinogen binding protein can be used in an ELISA test.

9.   DNA gene probe for the fibrinogen binding protein for ELISA tests.

10. Antibodies to the fibrinogen binding protein can be used to diagnose bacterial infections caused by S. aureus strains.

Table 1. ClfA proteins.

Protein mol. wt. apparent* predicted@		N-terminal sequence	ClfA residues
105kDa	57kDa	VGTLIGFGLL	23-32
75kDa	ND	GKIIGID	not related
55kDa	44kDa	MNQTSNETTFNDTNTV	143-157
42kDa	36kDa	AVAADAPAAGTDITNQLT	220-237

Native ClfA

190kDa	92kDa		

*   determined from migration on SDS-PAGE and Western blotting.

@   predicted from the amino acid sequence

ND   not determined.

Table 2. Inhibition of clumping with anti-ClfA sera.

Sera	Inhibiting concentration* (micro g)
N2	2.34
N3	2.34
Preimmune N2	>300.00
Preimmune N3	>300.00
C2	>300.00

*   Average of 3 experiments.

Table 3. Inhibition of clumping with lysates containing truncated ClfA proteins.

Lysate	Inhibiting concentration* (micro g)
pCF24	9.37
pCF25	>300.00
pCF24 Uninduced	>300.00
pCF25 Uninduced	>300.00
pCF27	>300.00
pCF28	>300.00
pCF29	>300.00
pCF30	>300.00
pCF31	9.37

*   Average of 3 experiments.

Table 4. The ability of lysates to block the inhibiting effect of anti-ClfA N2 sera on cell clumping.

Lysate	Blocking concentration* (micro g)
pCF24	1.17
pCF25	>75.00
pCF27	>75.00
pCF28	>75.00
pCF29	>75.00
pCF30	2.34
pCF31	2.34

* Average of 3 experiments.

Table 5. Experimental endocarditis

5	% infected	Newman	Newman clfA::Tn917	Newman clfA::Tn917 pCF16 clfA+
10	vegetation	84%	43%*	83%
	blood cultures	70%	30%*	50%
	spleen (x log CFU/g)	3.16	3.11	3.59

15 * p = 0.05 when compared to other groups

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